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Minimalist barcodes for sponges - A case study classifying African freshwater Spongillida

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Abstract:

African sponges, particularly freshwater sponges, are understudied relative to demosponges in most other geographical regions. Freshwater sponges (Spongillida) likely share a common ancestor; however, their evolutionary history, particularly during their radiation into endemic and allegedly cosmopolitan groups, is unclear.

Freshwater sponges of at least 58 species of 17 genera and four families are described from Central and Eastern Africa, but the diversity is underestimated due to limited distinguishable morphological features. The discovery of additional cryptic species is very likely with the use of molecular techniques such as DNA barcoding. The Royal Museum of Central Africa (MRAC, Tervuren, Belgium) hosts one of the largest collections of (Central) African freshwater sponge type material. Type specimens in theory constitute ideal targets for molecular taxonomy; however, the success is frequently hampered by DNA degradation and deamination, which are a consequence of suboptimal preservation techniques. Therefore, we genotyped African demosponge holotype material of the MRAC with specific short primers suitable for degenerated tissue and compare the results with the current, morphology-based classification. Our results demonstrate the utility of minimalistic barcodes for identification of sponges, potentially enabling efficient identification of individuals in taxonomic or metabarcoding studies, and highlight inconsistencies in the current freshwater sponge classification.

Keywords:
minimalist barcode, freshwater sponges, classification, DNA barcoding, Porifera, Spongillida.

Introduction:

Since the 1990s, molecular tools in sponge systematics, such as allozymes or comparative DNA sequence analyses, have revolutionized sponge classification and taxonomy at various hierarchical levels, from populations to species to phylum level (e.g., Kelly-Borges et al. 1991; Wörheide et al. 2012; Wörheide and Solé-Cava 2005). Classical sponge morphological as well as chemotaxonomic characters are considerably prone to environmental plasticity and homoplasy, and are therefore often unable to resolve relationships and boundaries among sponge lineages (Boury-Esnault 2006; Cárdenas and Rapp 2013; Erpenbeck et al. 2006; Erpenbeck and Van

Soest 2007; Maldonado et al. 1999). Results of molecular systematics studies have generated dramatic changes in poriferan systematics (Erpenbeck and Wörheide 2007, Voigt et al. 2012, Wörheide et al. 2012, Gazave et al. 2012, Redmond et al. 2013, Morrow and Cárdenas 2015). They have also provided us with a better understanding of sponge speciation and radiation (e.g., Blanquer and Uriz 2007, Reveillaud et al. 2010), and new tools forpecies delimitation (e.g., the Sponge Barcoding Project, www.spongebarcoding.org, Wörheide and Erpenbeck 2007).

A fundamental prerequisite for robust molecular evolutionary analyses is the use of taxonomically reliable DNA templates. Misidentified specimens skew every phylogenetic hypothesis independent of the algorithm and their mislabelled genomic traces can persist in public genetic repositories such as National Center for Biotechnology (NCBI) Genbank or European Nucleotide Archive (ENA) for a considerable time (Erpenbeck et al. 2015). The optimal taxonomic reference material for evolutionary analyses is therefore type material, particularly the holotype, which is usually consulted in morphological taxonomy or systematics, but rarely examined in sponge molecular studies (Erpenbeck et al. 2015). Although type material sequencing in molecular systematics is steadily increasing (Federhen 2015), usage is hampered by low DNA quality due to age and means of preservation. DNA in the *post-mortem* cell is subject to deterioration by oxidative and hydrolytic damage, DNA crosslinks, and nucleases that lead to fragmentation, inhibition of enzymatic reactions, and deamination-triggered sequence modifications (Hofreiter et al. 2001, Rizzi et al. 2012). Among these, fragmentation of DNA into pieces shorter than the envisaged marker is probably the primary obstacle for holotype amplification with standard markers, but should not hinder a molecular taxonomic comparison. Short DNA markers, so-called *minimalist DNA barcodes* (Hajibabaei et al. 2006), can facilitate genotype comparison with degenerated type material using the Sanger sequencing methods if other methods for full-length amplification fail. Such minimalist DNA regions, which are sufficiently short for amplification from fragmented DNA templates

but sufficiently variable for taxonomic distinction, are sequenced and analyzed (Cárdenas and Moore 2017).

African sponges, particularly freshwater sponges, are largely understudied, compared to sponges in most other geographical regions (Van Soest et al. 2012) despite taxon richness and potential ecological importance exceeding other sessile freshwater filter feeders (Manconi and Pronzato 2007). Recent revisions conclude that species richness and geographic distribution of African freshwater sponges is underestimated (Manconi and Pronzato 2008, 2009). Freshwater sponges constitute a monophyletic group (Order Spongillida) within the demosponges, however their evolutionary history is unclear, particularly during their radiation into either local endemics or widespread cosmopolitan species. Freshwater sponges of at least 58 species of 17 genera and four families are described from Central and Eastern Africa (Manconi and Pronzato 2009), but the diversity may be underestimated, as evident from analyses on other African freshwater invertebrates (e.g., Genner et al. 2007). New research campaigns in the Afrotropics continuously lead to new discoveries and descriptions of new species (Manconi et al. 2015, Manconi and Pronzato 2017). On the other hand, of a total of 58 species reported by Manconi and Pronzato (2009), 25 were recorded only one time as a type specimen from their type locality. Furthermore, some species are represented only in the literature and vouchers are not accessible for reinvestigation. Therefore, to date no comprehensive phylogeny for freshwater sponges has been published that could corroborate the currently morphology-based classification of freshwater sponges due to the lack of reliable material.

The Royal Museum of Central Africa (MRAC, Tervuren, Belgium) hosts one of the most important collections of (Central) African freshwater sponges and their type material, ranging from collection years 1913 to the 1970s (e.g., Brien 1973). In the current study we assessed the utility and potential of *minimalist DNA barcodes* for understanding freshwater sponge diversity. Specifically, we genotyped African type material of MRAC and other freshwater sponge specimens with specific markers for

degenerated tissue, and compared the results with the current, morphology-based classification.

Material and Methods

Preserved specimen material from Africa has been provided by the Royal Museum of Central Africa (Tervuren, Belgium), the Naturalis Biodiversity Center (Leiden, the Netherlands) and the Università degli Studi di Genova (Italy). Additional museum specimens were provided by the Bavarian State Collection of Zoology (ZSM, Germany) for testing the feasibility of *minimalist DNA barcode* amplification, in particular for amazonian sponges collected by Ernst Josef Fittkau 1961-1965 for his research on sponge-dwelling chironomids (e.g., Fittkau 1971). Five specimens were freshly collected from four crater lakes in southwestern Tanzania in 2011 (Lakes Ilamba, Itamba, Massoko and Kingiri; Malinsky et al. 2015) and immediately preserved in ethanol. A total of 40 specimens of 14 species and 14 Spongillida indet. were included in this study (see Table 1 for a complete specimen list).

DNA was extracted using the QiAmp Mini-Kit (Qiagen) following the manufacturer's protocol and alternatively by Phenol-Chloroform CTAB Technique (Aldrich and Cullis 1993), which are more suitable for long-preserved tissue than standard medium throughput methods (Vargas et al. 2012). The standard barcoding marker for demosponges (C-Region 28S rDNA and the "Folmer" partition of the CO1 mtDNA (Folmer et al. 1994) bear insufficient systematicresolution for freshwater sponges (see e.g., Erpenbeck et al. 2011); however, the Internal Transcribed Spacers (ITS) of the nuclear rDNA cistron has repeatedly shown to provide the highest species-level resolution for Spongillida (e.g., Itskovich et al. 2013b, 2015) with negligible amounts of intragenomic variability (Itskovich et al. 2017).

In a first step, amplification of the complete ITS region (i.e., ITS1 – 5.8S rDNA – ITS2) was attempted for all specimens using the primer ITS-RA2-fwd (5'-GTC CCT GCC CTT TGT ACA CA-3') in combination with ITS2.2-rvse (5'-CCT GGT TAG TTT

CTT TTC CTC CGC-3') as published in Wörheide (1998). Simultaneously, two minimalist barcoding markers were amplified for all specimens based on comparison of all available spongillid ITS sequences as published in NCBI Genbank: The first marker covers about 100 bp ranging from the 5.8S 3' region of the ITS2 5' region (referred to as *5.8S-ITS2* in the following); the second marker covers about 180 bp of the ITS2 3' region of the 28S5' region (referred to as *ITS2-28S* in the following, see Fig. 1). The 5.8S-ITS2 fragment was amplified using the following primers: 5.8_Freshies_1180_9f: 5'-GCA CGT CTG TCT GAG CGT CCG-3' (5.8S forward) in combination with ITS2_Freshies_1174_3r: 5'-GCT TCG CAC TTS AAG GGA CGC-3' (ITS2 reverse). The ITS2-28S fragment was amplified using the following primers: ITS2_Freshies_1176_5f: 5'-TTG CGC GTC GGG AAC TCG AC-3' (ITS2 forward) in combination with 28S_Freshies_1178_7r: 5'-GCT TAT TGA TAT GCT TAATT CAG C-3' (28S reverse). The 25 µL PCR mix consisted of 5 µL 5x green Go*Taq*® PCR Buffer (Promega Corp, Madison, WI), 4µL 25mM $MgCl_2$ (Promega Corp, Madison, WI), 2 µL 10mM dNTPs, 2 µL BSA (100µg/ml), 1 µL each primer (5µM), 7.8 µL water, 0.2µL GoTaq® DNA polymerase (5u/µl) (Promega Corp, Madison, WI) and 2 µL DNA template. The PCR regime comprised an initial denaturation phase of 94° C for 3 min followed by 35 cycles of 30 s denaturation at 94° C, 20 s annealing (45°C for the complete fragment; 52° C for 18S-ITS1; 55° C for ITS2-28S) followed by 60 s elongation at 72° C each and a final elongation at 72° C for 5 min. PCR products were purified with a Freeze-Squeeze Method (Thuring et al. 1975) before cycle sequencing using the BigDye-Terminator Mix v3.1 (Applied Biosystems) following the manufacturer's protocol. Both strands of the template were sequenced on an ABI 3730 automated sequencer. Sequences were basecalled, trimmed and assembled in CodonCode Aligner v 3.7.1.1 (www.codoncode.com). Poriferan origin of samples were checked with BLAST against NCBI Genbank (www.ncbi.nlm.nih.gov/genbank). Sequences are deposited in the ENA under accession numbers XXXXXXX and in the Sponge Barcoding Database (SBD, www.spongebarcoding.org). Sequences were verified by a second, independent set of extraction, amplification and sequencing as

means of contamination prevention. See supplementary data for specimen details, accession numbers and links to the SBD. Sequences were aligned with other available Spongillida sequences as published in NCBI Genbank (www.ncbi.nlm.nih.gov) using MAFFT (Katoh and Standley 2013) as implemented in Geneious 8.1.9 (Biomatters, http://www.geneious.com, Kearse et al. 2012) under default settings. Bayesian inference trees were reconstructed with MrBayes 3.2.6 under the most complex model (as overparameterizing does not negatively affect Bayesian inference results, Huelsenbeck and Ranala 2004), with two runs of four MCMCMC chains for 10,000,000 generations under monitoring convergence of the chains and stopped when the standard deviation of split frequencies fell below 0.01. Maximum-likelihood reconstructions were generated with PhyML (Guindon et al. 2010) as implemented in Geneious 8.1.9 under the same model and 100 bootstrap replicates. Trees are unrooted as the inclusion of non-spongillid outgroups inferred extremely long branches. All alignments, as well as input and output files have been deposited in an open access repository at https://github.com/PalMuc/XXX. The results of the phylogenetic analyses are compared with the current (morphology-based) classification following Manconi and Pronzato (2002) and the World Porifera Database (Van Soest et al. 2018).

Results and Discussion

For specimens of the “historic” sponge material (here defined as museum material collected before 1970), no complete ITS regions could be retrieved, independent of the preservation method, probably due to fragmentation of the DNA into pieces shorter than the marker length (ca. 500 bp). In turn, the *minimalist barcodes* 5.8S-ITS2 and ITS2-28S could be amplified for 32 out of 59 historic specimens, independent from their age or preservation method (see Fig. 2). DNA of freshly collected specimens was amplified with the *minimalist barcoding* primers, and the resulting sequences were identical with the corresponding region amplified with the full-length ITS primers (ITS-RA2-fwd & ITS2.2-rvse). Consequently, the *minimalist barcodes* are usable for

species identification of freshly collected specimens as well as to verify sequences published in databases such as NCBI Genbank or ENA. Their specific genotypes are usable as freshwater sponge barcode for unambiguous identification; their characteristic *sequence signatures*, i.e. motifs in the alignments present in the sequence of all members of a taxon but not in others (Gupta 1998), aid in freshwater sponge classification.

The final alignments (after merging of sequences from identical Genbank species to reduce alignment size) comprised 306 (5.8S-ITS2) and 168 (ITS2-28S) characters, respectively, with sequence lengths varying from 89bp to 126bp and 132bp to 230bp, respectively. The 106 (5.8S-ITS2) and 102 (ITS2-28S) taxa displayed total nucleotide differences of up to 59% (5.8S-ITS2) and 64% (ITS2-28S). The ITS2-28S minimalist barcodes are distinct for all species included in the data set, while lubomirskiid sequences published in NCBI Genbank as different species shared identical 5.8S-ITS2 minimalist barcoding regions (see Fig. 2). The underlying discrepancies in intra-lubomorskiid classification were noted earlier and already prompted for a revision of this family (e.g., Itskovich et al. 2015).

The sequences fell into distinct sequence signatures, mostly representing well-supported monophyletic groups (see Fig. 2). Support for deeper nodes is lacking, likely due to the high variability of the ITS fragments, which prevent unambiguous alignment among of all sequences. Nevertheless, the primary aim of *minimalist barcodes* in our approach is not the reconstruction of a phylogeny but the unambiguous classification of relevant (e.g., type) and challenging (e.g., historic) material to a taxon or lineage. We recover the following pattern relevant for African Spongillida classification:

The **Spongillidae** Gray, 1867 are a cosmopolitan family of (almost) entirely gemmular propagule-forming taxa. Spongillidae is the most specious (over 150 species on a total of ca. 240) and oldest, still valid, taxon erected from which all subsequent families were derived; Lubomirskiidae by Weltner in 1895 (see Rezvoi 1936); Potamolepidae by Brien in 1967; Metaniidae by Volkmer-Ribeiro in 1986 and Malawispongiidae by Manconi & Pronzato, in 2002. Spongillidae are here

represented by sequences published as *Ephydatia*, *Eunapius*, *Trochospongilla*, *Radiospongilla*, *Heterorotula, Nudospongilla* and *Spongilla*, and cannot be recovered as monophyletic (Fig. 2). This finding corroborates earlier molecular work demonstrating non-monophyly of this large freshwater sponge family (Addis and Peterson 2005, Meixner et al. 2007, Itskovich et al. 2008, 2013a, Erpenbeck et al. 2011), and implicates several Spongillidae lineages as founder for more endemic freshwater Spongillida. This suggests that further analyses including additional type material of Spongillidae are required.

Malawispongiidae Manconi & Pronzato, 2002 was erected in the last major revision of sponge genera, the Systema Porifera (Hooper and Van Soest 2002) as a new freshwater sponge family to host some lineages exclusively known from ancient lakes, except Lake Baikal. Each genus is endemic to a single ancient lake (type locality). This family was originally founded as the Spongillidae subfamily Globulospongillinae by Brien (1973) to accommodate gemmule-lacking ancient lake taxa before it was promoted to family level (Racek 1974) and (after declaration as *nomen nudum*) renamed to Malawispongiidae (Manconi and Pronzato 2002). Malawispongiidae comprises five genera with a notably disjunct distribution in Africa, Asia, and Europe. Its genera are strictly endemic to their particular ancient lakes and predominantly monotypic: *Cortispongilla* Annandale, 1918 (1 species: *C. barroisi* Topsent, 1892 in Lake Kinneret), *Malawispongia* Brien, 1972 (1 species: *M. echinoides* Brien, 1972 in Lake Malawi), *Ochridaspongia* Arndt, 1937 (2 species, *O. rotunda* Arndt, 1937 and *O. interlithonis* Gilbert & Hadzische, 1984 in Lake Ochrid), *Pachydictyum*Weltner, 1901 (1 species, *P. globosum* Weltner, 1901 in Lake Posso), and *Spinospongilla* Brien, 1974 (1 species, *S. polli* Brien, 1974 in Lake Tanganyika). From a biogeographic point of view this highly disjunct distribution, with ranges restricted only to the lacustrine type locality, makes monophyletism of this taxon unlikely. On the other hand morphological analyses suggest a very close parallelism of diagnostic traits for malawispongiid taxa, clearly separating them from the other freshwater sponge lineages.

Our current study provides molecular data for classification of the African malawispongiids *Malawispongia* and *Spinospongilla* based on type material (Fig. 2, all malawispongiid taxa in red). The *M. echinoides* holotype (MRAC1426), the paratype (MRAC1427) and additional specimens were successfully sequenced for both *minimalist barcoding* regions and form a monophyletic clade with a distinct sequence signature with non-malawispongiids and distant from *Spinospongilla polli* (holotype MRAC1413). Sequence differences among the *M. echinoides* type specimens indicate intraspecific variability in hypervariable positions and cannot be caused by deamination artefacts, as obvious by their similar positions among the specimens. Furthermore, a specimen of *Ochridaspongia rotunda* (FW695), type species of *Ochridaspongia,* likewise displays no close relationship to other malawispongiids, but a close relationship to *Ephydatia fluviatilis* (Spongillidae), corroborating earlier, independent findings with CO1 (Meixner et al. 2007). In the same publication, specimens identified as *Pachydictyum globosum* were found as distantly related to *Ochridaspongia* (Meixner et al. 2007). However, these results await verification by additional, independent data, preferably from type material. Likewise, ITS of specimens published as *Cortispongilla barroisi* have been found identical to *Ephydatia fluviatilis* (Itskovich et al. 2013b, sequences also incorporated in Fig. 2), and likewise await verification by type material.

In combination with results from earlier (although not yet holotype-verified) molecular analyses (e.g. Meixner et al. 2007, Itskovich et al. 2013b), our preliminary results based on the minimalist barcodes indicate that Malawispongiidae in current composition is not monophyletic. The placement of *Ochridaspongia, Cortispongilla* and *Pachydictyum* holotypes (currently under investigation) will indicate whether malawispongiids constitute a polyphyletic assemblage with disjunct distribution, combined by primarily negative characters (the absence of gemmules and microscleres), a frequent practice when morphological characters were insufficient (see Boury Esnault and Volkmer Ribeiro 1992). Spongillidae, however, can display a wide range of ecomorphic variants including spicule composition and morphology

leading to overestimation of collected species; formation of gemmules appears unnecessary in deep, stable lakes and facilitating a loss of this discriminating apomorphy (Manconi and Pronzato 2007, see discussion in Itskovich et al. 2013b). Therefore, as long as as the phylogenetic placements of *Cortispongilla* and *Pachydictyum* await verification, we refrain to propose abandoning Malawispongiidae and reclassification of their genera.

The evidence of being all inhabitants of ancient lakes could be considered the common driver of a morphofunctional adaptive trend involving the morphological and anatomical traits. Malawispongiid characters, such as spiny monoaxial megascleres, lack of microscleres and/or gemmules, are common among freshwater sponges and shared with the only other endemic ancient lake family the Baikalian Lubomirskiidae, which are well studied at morphological and molecular level.

Potamolepidae Brien, 1967 have an Afrotropical, Neotropical, Australian, Oriental and Nearctic distribution (Manconi and Pronzato 2002, Manconi et al. 2012, Copeland et al. 2015).. Our present study comprises sequences of all Afrotropical genera (*Echinospongilla* Manconi & Pronzato, 2002, *Oncosclera* Volkmer-Ribeiro, 1970, *Potamolepis* Marshall, 1883, *Potamophloios* Brien, 1970). The taxa fall into two distinct clades (Fig. 2), with yet unresolved relationship to each other, however, not rejecting potamolepid monophyly: 1) A clade with members of *Potamolepis*, i.e., *P. marshalli* Burton, 1938 (MRAC144, holotype), and *P. pechueli* Marshall, 1883 (MRAC195). 2) A second clade comprising representatives of all remaining (Afrotropical) potamolepid genera with a supported sequece signature. We recover the type specimens of *Potamophloios songoloensis* Brien, 1969 (holotype MRAC1317) and *Potamophloios hispida* Brien, 1969 (holotype MRAC1318) in a clade together with a a Genbank-published *Echinospongilla brichardi* (Brien, 1974) and three not further determined *Oncosclera* and *Potamolepis* sp. supporting their close relationship in this family (Manconi and Pronzato 2002). *Spinospongilla polli* Brien, 1974 (holotype MRAC1431) currently classified into Malawispongiidae, is a further species falling into

Potamolepidae (see Fig. 2, blue field). This monotypic genus, endemic to Lake Tanganyika, displays a morphological character suite (monoaxial megascleres, no microscleres, no gemmules observed) like most malawispongiids, thus preventing unambiguous placement within the Spongillidae with morphological methods. Manconi and Pronzato (2009), however, remark the need of further investigations for *Spinospongilla polli* and all *Potamophloios* and *Potamolepis* species particular due to the lack of gemmules in most type specimens. Our *minimalist barcodes* now provide a reliable measurement to identify other material and draw conclusions on gains and losses in gemmule production in potamolepid species.

Metaniidae Volkmer-Ribeiro, 1986 falls into two distinct clades representing Afropaleotropical and Neotropical specimens respectively (Fig. 2, orange field). Resolution power of the minimalist barcodes, however, is insufficient to provide a metaniid phylogeny in this approach. The minimalist barcodes successfully amplified paratypes of *Metania lissostrongyla* Burton, 1938 (MRCA134 and MRCA197), which is currently synonymized as *Metania pottsi* (Weltner, 1895) (Castello-Branco et al. 2015, Manconi et al. 2015,). Both specimens are distant from MRCA1006, a specimen identified as *Potamolepis schoutedeni* Burton, 1938 (Manconi and Pronzato 2009, Manconi et al. 2015) and currently synonymized as *Metania pottsi* (Weltner, 1895) (Silva and Volkmer-Ribeiro 1998). The genus and family transfer is now fully supported by molecular data. However the junior synonymy of *P. schoutedeni* and *M. lissostrongyla* under *M. pottsi* is not supported by molecular data. Indeed the first species resulted close to the Neotropical metaniidluster and not to the Afrotropical species, as expected, considering the fact that both the type localities are near Leopoldville (Fig. 2). Our current data clearly demonstrate that both lineages are distinct rejecting the current classification into one species.

Several samples of yet **undetermined sponge material** could be classified with the minimalist barcodes. The material from the Fittkau collection (collection nummers ZSM 20xxxxxx) was partially identified by Cecília Volkmer-Ribeiro and

several, yet unidentified specimens from this collection displayed identical minimalist barcodes and thus provided a preliminary first identification. Equally, specimens from material collected from southern Tanzania crater lakes (SNSB-BSPG.GW2354 - SNSB-BSPG.GW2358) could be identified with the aid of the minimalist barcodes, in this cases as *Metania pottsi*, *Malawispongia echinoides*, and further potamolepid taxa.

Our results display a high suitability for the minimalist barcodes in the understanding and unraveling of freshwater sponge classification. We demonstrate that taxonomically informative *minimalist barcodes* can be amplified from historic collection material (incl. types), which were stored with preservation unfavourable for the extraction of high molecular weight DNA. The barcodes have a sufficient shortness for amplification of highly fragmented DNA, and a sufficient length for confident identification of many samples to species level. Nevertheless, the barcodes are too short to harbor supported phylogenetic signal for unravelling the deeper Spongillida phylogeny. However, additional freshly collected or well-preserved material for molecular studies can now be unambiguously identified with holotype minimalist barcodes. A comprehensive reference library is under construction as part of the Sponge Barcoding Project (www.spongebarcoding.org). For African (and other) freshwater sponge species, which are for a considerable part based on monotypic genera and single specimen species and simultaneously massively understudied, the *minimalist barcodes* provide an valuable marker for the assessment of biodiversity and verification of the classification.

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Table 1. List of species sequenced in this study

Species	Museum Registration	Type	Year	Storage	Status
Drulia browni (Bowerbank, 1863)	ZSM 20020195	no	1962	dry	valid
Malawispongia echinoides Brien, 1972	MRCA 1427	Paratype	1971	EtOH	valid
Malawispongia echinoides Brien, 1972	MRCA 1426	Holotype	1971	EtOH	valid
Malawispongia echinoides Brien, 1972	ZMA POR9581	no	1991	EtOH	valid
Malawispongia echinoides Brien, 1972	SNSB-BSPG.GW2355	no	2011	EtOH	valid
Metania lissostrongyla Burton, 1938	MRCA 134	Paratype	1937	dry	*Metania pottsi* (Weltner, 1895)
Metania lissostrongyla Burton, 1938	MRCA 197	Paratype		EtOH	*Metania pottsi* (Weltner, 1895)
Metania reticulata (Bowerbank, 1863)	ZSM 20020206	no		dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020207	no	1961	dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020209	no		dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020211	no	1965	dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020218	no	1961	dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020220	no	1962	dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020222	no	1988	dry	valid
Metania reticulata (Bowerbank, 1863)	ZSM 20020201	no	1961	dry	valid
Ochridaspongia rotunda Arndt, 1937	FW695	no	2005	dry	valid
Potamolepis marshalli Burton, 1938	MRCA 144	Holotype		dry	valid
Potamolepis pechueli Marshall, 1883	MRCA 195	no	1937	EtOH	valid
Potamolepis schoutedeni Burton, 1938	MRCA 1006	no	1945	dry	*Metania pottsi* (Weltner, 1895)
Potamophloios hispida Brien, 1969	MRCA 1318	Holotype	1966	dry	valid
Potamophloios songoloensis Brien, 1969	MRCA 1317	Holotype	1966	dry	valid
Spinospongilla polli Brien, 1974	MRCA 1431	Holotype	1974	EtOH	valid
Spongilla lacustris (Linnaeus, 1759)	ZSM 20020172	no	1906	EtOH	valid
Spongilla moorei Evans, 1899	MRCA 413	no	1913	EtOH	*Nudospongilla moorei* (Evans, 1899)
Spongilla moorei Evans, 1899	MRCA 433	no	1913	EtOH	*Nudospongilla moorei* (Evans, 1899)
Trochospongilla horrida Weltner, 1893	ZMA POR20942	no	2008	EtOH	valid
Spongillida indet	SNSB-BSPG.GW2357	no	2011	EtOH	
Spongillida indet.	SNSB-BSPG.GW2356	no	2011	EtOH	
Spongillida indet.	ZSM 20020225	no	1963	dry	
Spongillida indet.	ZSM 20020230	no		dry	
Spongillida indet.	ZSM 20020232	no	1962	dry	
Spongillida indet.	ZSM 20020237	no	1961	dry	
Spongillida indet.	ZSM 20020238	no		dry	
Spongillida indet.	ZSM 20020239	no	1965	dry	
Spongillida indet.	ZSM 20020228	no	1965	dry	
Spongillida indet.	ZSM 20044492	no	1996	dry	
Spongillida indet.	ZSM 20150098	no		EtOH	
Spongillida indet.	SNSB-BSPG.GW2358	no	2011	EtOH	
Spongillida indet.	SNSB-BSPG.GW2354	no	2011	EtOH	
Spongillida indet.	ZMA POR16905	no	2001	EtOH	

Figure Legends:

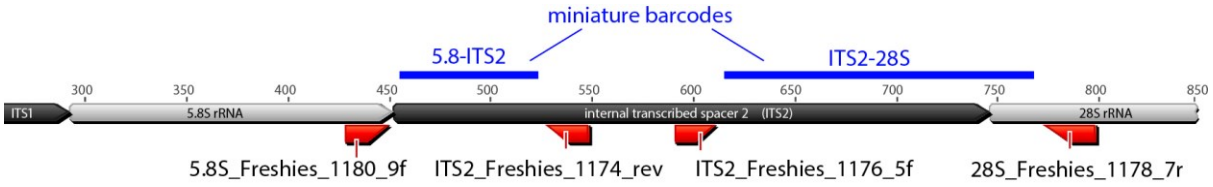

Figure 1: Position of the minimalist barcode primers in the rDNA gene cluster with respect to *Eunapius* sp. (EF151946).

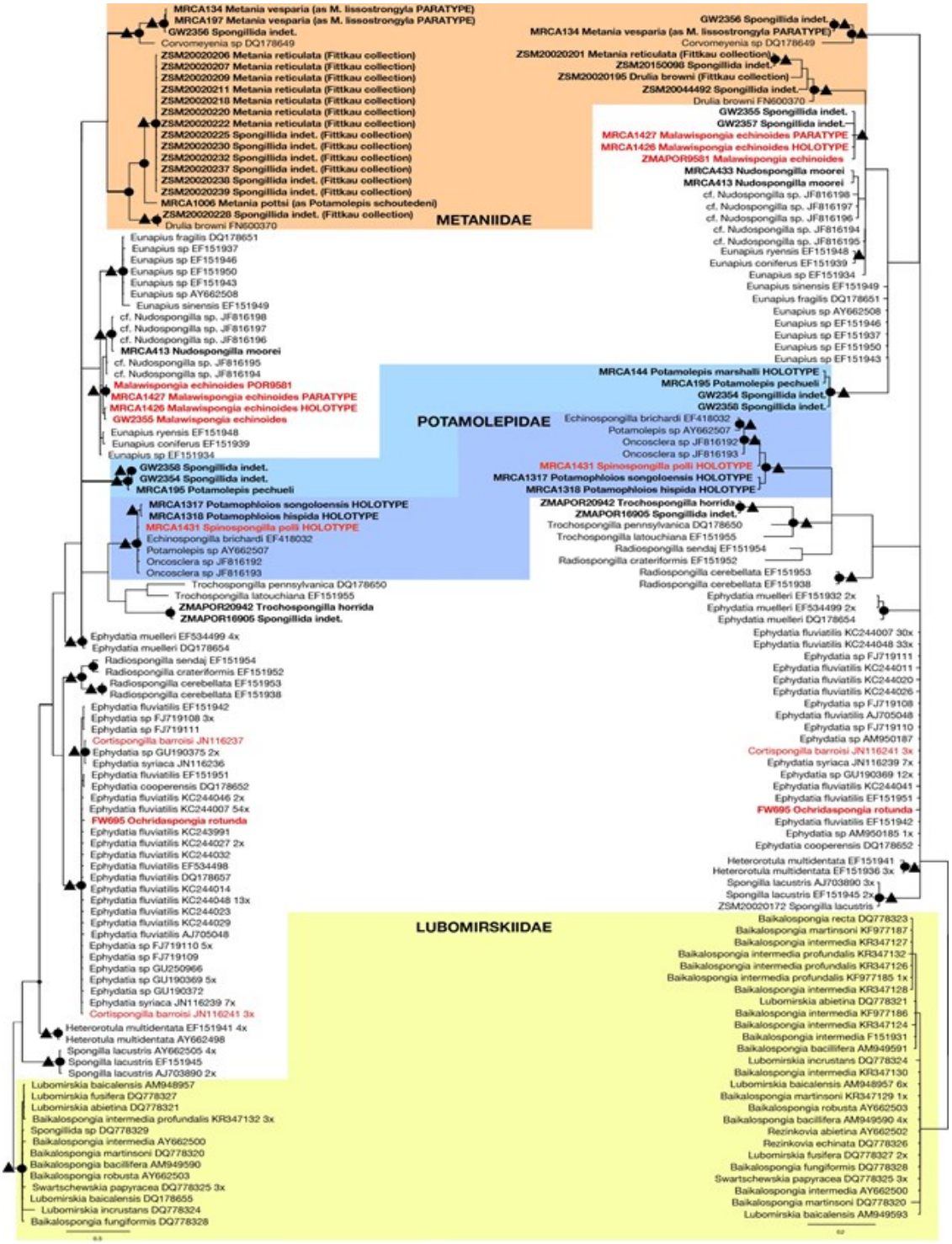

Figure 2: Unrooted bayesian inference phylogram of the 5.8S-ITS2 (right) and ITS2-28S (left) minimalist barcoding regions. New sequences are in boldface preceded by their collection numbers; taxon names in regular font are published in Genbank, followed by the accession number and occasionally the number of additional conspecifics with this sequence (e.g. 3x). Malawispongiid taxa are given in red taxon

names. Lubomirskiidae (yellow), Potamolepidae (blue), and Metaniidae (orange) are underlaid in coloured boxes. All other taxa are Spongillidae. Black dots on the branches indicate Posterior probabilities in significant range (>0.95). Black triangles indicate Maximum-likelihood bootstrap support > 70.